Modulation of cytochrome oxidase activity by inorganic and organic phosphate

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The activity of cytochrome oxidase reconstituted into phospholipid vesicles has been studied as a function of orthophosphate, ATP and inositol hexakisphosphate concentrations. The respiratory-control ratio was found to be quite sensitive to these compounds and was inversely related to the anion concentration. This effect is related to a phosphate-dependent decrease in the rate constant for ferrocytochrome *c* oxidation observed in the presence of ionophores. The data cannot be interpreted simply on the basis of ionic strength, which is known to limit cytochrome *c* binding to cytochrome oxidase, since cytochrome oxidase-containing vesicles responded differently to phosphate depending on the energization state of the phospholipid membrane.

INTRODUCTION

Electron transfer from ferrocytochrome *c* to molecular oxygen via cytochrome oxidase is a complex process involving several redox reactions: (i) electron-transfer between ferrocytochrome *c* and cytochrome *a*; (ii) a fast internal electron equilibration with the e.p.r.-visible Cu_a ; (iii) a rate-limiting step of the overall reaction involving the transfer of electrons to the binuclear cytochrome a_3 - Cu_a site; and (iv) a rapid four-electron transfer to molecular oxygen to yield water. Reaction (i) has been followed in a stopped-flow apparatus (both as ferrocytochrome *c* oxidation or ferricytochrome *a* reduction) under a number of conditions involving, in most cases, phosphate in the medium and low cytochrome *c* concentrations (Gibson *et al.*, 1965; Andreasson *et al.*, 1972; Wilson *et al.*, 1975). When cytochrome oxidase is reconstituted into artificial phospholipid vesicles ('cytochrome oxidase-containing vesicles', COV), the overall catalytic efficiency is increased by a factor of 40–50, owing to phospholipid activation being fully developed in the presence of ionophores (which release respiratory control) (Sarti *et al.*, 1983). In COV the time course of oxidation of reduced cytochrome *c* in air and its associated redox-linked proton translocation (Wikstrom, 1977; Sarti *et al.*, 1985) depend on the development of an electrochemical gradient and on the presence of ionophores (Brunori *et al.*, 1985). In the present paper we report a study intended to explore the effect of inorganic and organic (ATP and InsP_6) phosphate on the catalytic activity of coupled and uncoupled COV.

The results indicate that phosphate dramatically affects cytochrome *c* oxidation by COV with high RCR (= 10–20) in the uncoupled state, thereby lowering the observed RCR. A novel phosphate-binding site on cytochrome oxidase is suggested to modulate electron-transfer activity, although the modality of its action with respect to the energized membrane is still unclear.

MATERIALS AND METHODS

Enzymes and chemicals

Cytochrome *c* oxidase was prepared as described by Yonetani (1961). The final suspending buffer contained 0.2% sodium cholate. Cytochrome *c* (Type VI), valinomycin, CCCP, ATP and InsP_6 were from Sigma Chemical Co. All other chemicals were of analytical grade.

Phospholipid-vesicle preparation

COV were prepared by the method of Casey *et al.* (1983) by using L- α -phosphatidylcholine (type II-S from Sigma) and were tested for both the RCR (Hinkle *et al.*, 1972) and orientation. The cytochrome oxidase molecules with cytochrome *a* facing the external medium (Sarti *et al.*, 1983) were found to account for 85% of the total molecules both in the presence and in the absence of phosphate. RCR ranged between 10 and 20 for different COV preparations. This variability depends on a number of different factors, including the age of the cytochrome *c* oxidase.

Rapid-mixing measurements

The experiments were carried out with a Durrum-Gibson stopped-flow apparatus equipped with a thermostatically controlled (20 °C), 2 cm-light-path, observation chamber. Typically, COV containing 1.25 μM oxidase functional units, suspended in 0.1 M-Hepes buffer, pH 7.3, and in the presence or the absence of 5 μM -valinomycin and 10 μM -CCCP, were mixed with 20 μM -ferrocytochrome *c* (reduced with sodium dithionite and chromatographed on Sephadex G-25 to remove excess reductant) dissolved in the same buffer. Phosphate was added to the cytochrome *c*-containing syringe at the concentrations indicated in Figure legends. Optical spectra were recorded on a thermostatically controlled Cary 219 spectrophotometer.

Abbreviations used: COV, cytochrome oxidase-containing vesicles; InsP_6 , inositol hexakisphosphate; RCR, respiratory-control ratio; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Fig. 1. Effect of phosphate concentration on the time course of ferrocytochrome *c* oxidation in air catalysed by COV

COV ($1.25 \mu\text{M}$ functional unit) were mixed with $20 \mu\text{M}$ -ferrocytochrome *c* in the absence (a) or presence (b) of ionophores (for details, see the Materials and methods section). Phosphate was added to the cytochrome *c*-containing syringe as follows: trace 1, no addition; trace 2, 10 mM; trace 3, 50 mM; trace 4, 100 mM; trace 5, 200 mM. Arrows on the vertical axis indicate that absorbance change recovered on increasing phosphate concentration. The data are represented in terms of semi-logarithmic plots of cytochrome *c* oxidation (monitored at 550 nm).

RESULTS

The time course of ferrocytochrome *c* oxidation catalysed by COV with a high RCR (10–20) has been shown to be biphasic in the absence of ionophores and to exhibit a simple exponential behaviour in their presence (Brunori *et al.*, 1985). This complex behaviour has been interpreted in terms of a conformational model of cytochrome oxidase reconstituted into phospholipid vesicles involving two kinetically and bioenergetically relevant distinct states which are in an equilibrium poised by the magnitude of the transmembrane electrical gradient, $\Delta\psi$.

This biphasic behaviour is not altered qualitatively by changing phosphate concentration (Fig. 1a) or ATP and InsP_6 concentrations (results not shown). The recovery of absorbance change determined in the stopped-flow apparatus for the fast initial phase is progressively increased at higher phosphate concentrations, in line with the decreased bimolecular rate constant for cytochrome *c* binding in the presence of phosphate (Wilson *et al.*, 1975) or other anions. The magnitude of increased

absorbance recovery is shown in Fig. 1 by the vertical arrow. The subsequent exponential phase, which corresponds to a steady state (rate-limited by the efficiency of electron transfer from cytochrome a_3 - Cu_a to the cytochrome a_3 - Cu_a site), shows a complex behaviour in the absence of valinomycin and CCCP. At low phosphate concentrations, activity is enhanced, whereas at higher concentrations it is inhibited, thus displaying a maximum of activity (see below).

In the presence of ionophores (valinomycin and CCCP) (Fig. 1b) the time course of ferrocytochrome *c* oxidation conforms to a simple exponential process whose rate constant closely parallels that of the initial rapid phase seen in the absence of ionophores (Fig. 1a) (Brunori *et al.*, 1985). In this case, however, the effect of phosphate (and ATP or InsP_6) is dramatic, and the rate constants for cytochrome *c* oxidation are inversely related to phosphate concentration and do not go through a maximum, as is observed in the absence of ionophores. This result is an indication of a differential reactivity of COV towards phosphate that depends on the energization state of the phospholipid membrane.

Detergent-solubilized cytochrome oxidase responds to organic and inorganic phosphate in a qualitatively similar manner to COV in the absence of ionophores. This is shown in Figs. 2(b) and 2(a) respectively. The plots are represented in terms of the ratio k'/k_0 , i.e. the rate constant of ferrocytochrome *c* oxidation at a given phosphate concentration normalized to the corresponding rate in its absence. At low phosphate concentrations there is a clear enhancement of the catalytic activity of cytochrome oxidase (independent on environment, i.e. detergent or vesicles), followed by a decrease in the rate constant at higher phosphate concentrations, as shown in Fig. 1. Similar results were obtained for both ATP and InsP_6 , as shown in Fig. 2, although for InsP_6 the activation process was much less evident in detergent-solubilized cytochrome oxidase (Fig. 2b) and very pronounced in COV (Fig. 2a).

A direct consequence of the results presented in Figs. 1 and 2 is that the RCR of COV becomes a function of inorganic- and organic-phosphate concentration. This is clearly shown in Fig. 3, in which the pseudo-first-order rate constants for ferrocytochrome *c* oxidation are plotted as a function of phosphate concentration in the presence of (k_{+1}) and absence (k_{-1}) of ionophores. The RCR, i.e. the ratio of these two rate constants, is critically dependent on phosphate concentration (insets to Fig. 3), and its fall is correlated with the much more efficient inhibition of k_{+1} with respect to k_{-1} .

Fig. 4 demonstrates direct binding of InsP_6 to detergent-solubilized cytochrome oxidase as determined by difference optical spectroscopy. This experiment was done in the absence of ferrocytochrome *c* or other reductants and yields a small, but measurable, spectral shift induced by phosphate, ATP (results not shown) and InsP_6 . The data indicate the availability of phosphate-binding sites on cytochrome oxidase, which may be relevant to the kinetic data reported above, since binding of organic phosphate can be detected at concentrations below $100 \mu\text{M}$.

DISCUSSION

The effects introduced by phosphate, ATP and InsP_6 described here are not simple to interpret. Early studies

Fig. 2. Dependence of the pseudo-first-order rate constant for ferrocytochrome *c* oxidation by cytochrome oxidase on inorganic- and organic-phosphate concentration

(a) COV in the absence of ionophores; (b) detergent-solubilized cytochrome oxidase. All experimental conditions were as in Fig. 1. Arrows indicate the appropriate abscissa scale. $\circ$, Phosphate; $\triangle$, ATP; $\bullet$, InsP_6 . The ordinate is given in terms of the ratio between the observed rate constant at a given phosphate concentration (k') and the rate observed in its absence (k_0).

Fig. 3. Dependence of the RCR on inorganic- and organic-phosphate concentration

All experimental conditions were as for Fig. 1. The pseudo-first-order rate constants for ferrocytochrome *c* oxidation by COV in the presence ($\circ$) and absence ($\blacksquare$) of ionophores are plotted as a function of phosphate (a), ATP (b) and InsP_6 (c) concentration. The ratio of the rate constants in the presence of ionophores to that in its absence (RCR) is plotted as a function of phosphate, ATP and InsP_6 concentration in the corresponding insets.

by Ferguson-Miller *et al.* (1976) demonstrated that the steady-state kinetics (measured polarographically) of cytochrome *c* oxidation by cytochrome oxidase, either in Keilin-Hartree particles or detergent-solubilized, were critically dependent on the nature of the anion present in the assay medium. The high-affinity phase for cytochrome *c* oxidation ($K_d = 10^{-8}$ M) was almost completely abolished by phosphate and ADP, and only one phase was detected with ATP (see Figs. 5 and 6 of Ferguson-Miller *et al.*, 1976). Moreover, the binding of cytochrome *c* to cytochrome oxidase, as determined by equilibrium gel-filtration experiments (Ferguson-Miller *et al.*, 1976) on Sephadex G-75, was severely impaired in the absence of nucleotides and/or phosphate.

The intrinsic catalytic efficiency of cytochrome oxidase is (at high ionic strength, i.e. 0.05 M) due to the rate-limiting step in the overall process, i.e. the transfer of electrons from cytochrome *a*- Cu_a to the binuclear cytochrome *a*₃- Cu_a site (Brunori *et al.*, 1979). Cytochrome *c* binding cannot become rate-limiting, even at high ionic strength, since the bimolecular rate constant under these conditions (e.g. 0.1 M-phosphate, pH 7.4) is two orders of magnitude lower than the value obtained at lower ionic strength, which approaches the diffusion-controlled limit of $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. Therefore cytochrome *c* binding is still much faster than the rate of internal electron transfer to the binuclear site. Moreover, since the equilibrium constant for cytochrome *c* binding

Fig. 4. Binding of InsP_6 to detergent-solubilized cytochrome oxidase

Cytochrome oxidase concentration was $10 \mu\text{M}$ in 10 mM-Hepes, pH 7.5, containing 0.5 % Tween 80. The difference spectrum was obtained by addition of $200 \mu\text{M}$ - InsP_6 to the sample cuvette and an equivalent volume of buffer in the reference cell, after registration of the baseline. The temperature was 20°C . Similar results were obtained with P_i and ATP.

(which includes the electron-transfer event within the collisional complex) is close to unity in neutral 0.1 M-phosphate buffer (Greenwood *et al.*, 1976), the ferri-cytochrome *c* off rate also cannot introduce a serious rate limit (Brzezinski & Malmstrom, 1986).

The most interesting of the present findings is the observation that the RCR is an inverse function of phosphate concentration. The RCR is commonly determined by measuring the rate of oxygen consumption by a preparation of COV in the presence and absence of ionophores (Hinkle *et al.*, 1972). The values measured by the polarographic method closely match those obtained by stopped-flow techniques monitoring the oxidation of cytochrome *c* at 550 nm (Sarti *et al.*, 1983, 1985). Cytochrome *c* oxidation by COV is inhibited by phosphate much more efficiently in the presence of ionophores (Fig. 1b) than in their absence (Fig. 1a). As a consequence, the rate constant for cytochrome *c* oxidation in the presence of ionophores, k_{+1} , decreases much more rapidly than the rate in their absence, k_{-1} , and this therefore leads to a dramatic decrease of the observed RCR. This finding is not due to scrambling of the orientation of cytochrome oxidase in vesicles as determined independently by the *NNN'*-tetramethyl-*p*-phenylenediamine-ascorbate assay (Sarti *et al.*, 1983) in the presence of phosphate. Moreover, the reorganization rate of cytochrome oxidase with respect to vesicles is expected to be much lower than the time scale of the present experiments. If experiments were to be conducted in the presence of 50 mM-phosphate, a 50 % underestimate (see Fig. 3) of the RCR would result, although the relative orientation and incorporation of cytochrome oxidase are virtually unchanged.

The inhibition kinetics of COV observed at high anion concentrations can be interpreted on a twofold basis. (i) The bimolecular binding process of cytochrome *c* on cytochrome oxidase solubilized by detergents is known to be an ionic-strength-dependent phenomenon. Accordingly, the bimolecular rate constant decreases as

a function of ionic strength (Wilms *et al.*, 1981). Apparently, this overall behaviour is also observed in COV. As a matter of fact, higher absorbance recovery is obtained (Fig. 1), and ATP and InsP_6 , which bear higher numbers of negative charges than does phosphate (at a given pH), exert inhibition at much lower concentrations (Figs. 2 and 3). The oxidation of the first 2 mol of ferri-cytochrome *c*/mol of cytochrome oxidase functional unit is, however, still faster than the rate-limiting step measured during turnover under all the conditions explored. (ii) Phosphate, and anions in general, interfere with the internal monomolecular electron-transfer step which conveys electrons to the binuclear site. This has been already described for soluble cytochrome oxidase in previous reports (Wilson *et al.*, 1980; Wilms *et al.*, 1981), and the present results suggest that the same phenomenon may also occur in COV. The binding site for anions on cytochrome oxidase is not known. The binuclear site may be a candidate only if anions entered the vesicle interior sufficiently rapidly (see below). However, given the highly charged nature of either phosphate, ATP or InsP_6 at pH 7.3, the permeability of the liposomal membrane to these anions is quite low, precluding their passage within the time scale of the present experiments. It therefore appears that the phosphate-binding site(s) resides on the outer aqueous phase of the vesicle, which corresponds, topologically, to the intermembrane space of mitochondria. Montecucco *et al.* (1986) actually demonstrated specific ATP binding to cytochrome oxidase by use of a photoactivatable ATP analogue. Nucleotide-binding sites were thus found to reside on nuclear-coded subunits (IV and VIII). Both of these polypeptides bear one transmembranous segment, and thus it is not possible, at this stage, to localize the site of modification to either of the aqueous mitochondrial compartments. A possible complication may arise from the finding that hydrophilic protein-modifying reagents label subunit IV only in the *N*-terminal portion of the polypeptide and that the *N*-terminus is exposed to the matrix space of mitochondria (Malatesta *et al.*, 1983) or the internal space of phospholipid vesicles (Zhang *et al.*, 1985). However, these findings do not preclude ATP binding from the cytoplasmic space.

A point which still remains to be explained is the enhancement of activity at low phosphate concentrations. This is only seen in the case of detergent-solubilized cytochrome oxidase or of COV in the absence of ionophores (Fig. 2), but not after their addition. A similar effect has been described in previous reports (Wilson *et al.*, 1980; Smith *et al.*, 1981; Kadenbach, 1986) and also in the case of superoxide dismutase (Fee *et al.*, 1986). The system behaves as if the accessibility of phosphate to its binding site on oxidase were modulated by the electrochemical gradient across the liposomal membrane. Although ATP is synthesized in the matrix space, it is rapidly transported outside this space by the ATP-ADP translocator (Nicholls, 1982), which is located in the inner membrane. Given that the steady-state ratio $[\text{ATP}]/[\text{ADP}] \cdot [\text{P}_i]$ outside the mitochondrion is estimated to be 10 times the value in the matrix space (Klingenberg, 1970) and that the concentration of ATP in living cells is in the millimolar range (Lehninger, 1975), it is not impossible that modulation of cytochrome oxidase activity by ATP and/or other nucleotides may actually take place *in vivo*.

Recently Bisson *et al.* (1987) have found that ATP can

protect cytochrome oxidase from enzymic inactivation by a water-soluble carbodi-imide, a clear indication of an ATP-induced conformational transition. This finding correlates well with the spectroscopic shift in visible spectrum of cytochrome oxidase induced by phosphate, ATP and InsP_6 shown in Fig. 4.

The differential reactivity of COV to phosphate, anions and polyanions depending on the 'electrochemical' status of the phospholipid membrane may be an indication of the existence of membrane-potential-sensitive conformational transitions of cytochrome oxidase, which in turn may also reflect a possible mechanism for the control of cytochrome oxidase in the living cell.

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